ORIGINAL ARTICLE

Role of *FOXP3* Expression and Serum Vitamin D and C Concentrations When Predicting Acquisition of Tolerance in Infants With Cow's Milk Allergy

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■ **Abstract**

Background: Treg cells and dietetic factors may play a significant role in the natural acquisition of tolerance in children with cow's milk allergy (CMA). The best marker for Treg lymphocytes is the transcription factor forkhead boxP3 (*FOXP3*).
Objective: We examine the relationship between *FOXP3* mRNA expression and serum concentrations of vitamins D and C and the development of different phenotypes of tolerance in children with CMA.
Material and methods: The study group comprised 138 infants with CMA and 101 healthy infants. All children underwent oral food challenge, first with an extensively heated milk product and then with unheated products. *FOXP3* mRNA expression and serum vitamin C and D concentrations were evaluated.
Results: At 2 years of life, 54 children (39.1%) still had CMA, 43 (31.2%) were unheated milk–reactive and heated milk–tolerant, while 41 (29.7%) had outgrown their allergy. The mean (SD) level of *FOXP3* expression in the study group was 2.07 (1.23), which was lower than the control group value of 2.98 (1.52) (*P*<.001). A value below 1.45 indicated allergy. The mean serum level of vitamin D in the study group was lower than in the control group (29.67 [7.09] vs 33.35 [4.13] ng/mL; *P*<.001). No significant differences were found in mean serum vitamin C content.
Conclusions: Increased *FOXP3* mRNA expression can predict faster acquisition of tolerance in infants with CMA. These children have lower serum vitamin D levels than healthy children. No relationship was found between the natural history of CMA and serum vitamin C concentration.
Key words: Food allergy. *FOXP3*. Tolerance. Children. Vitamin D. Vitamin C. Cow's milk.

■ **Resumen**

Antecedentes: Las células Treg y los factores dietéticos pueden desempeñar un papel importante en la adquisición natural de tolerancia en niños con alergia a la leche de vaca (CMA). El mejor marcador de linfocitos Treg es el factor de transcripción Forkhead box P3 (FOXP3).
Objetivos: El artículo examina la relación entre la expresión de mRNA específico para FOXP3 y la concentración sérica de vitaminas D y C, así como el desarrollo de diferentes fenotipos de tolerancia en niños con CMA.
Material y métodos: El grupo de estudio estaba compuesto por 138 bebés con CMA y 101 sanos. Todos los niños tomaron primero un producto lácteo hervido vía oral, y posteriormente productos lácteos sin calentar. Se evaluó la expresión de ARNm para FOXP3 y la concentración sérica de vitamina C y D.
Resultados: A los dos años de vida, 54 (39,1%) de los niños aún mostraban CMA, 43 (31,2%) eran reactivos a la leche sin calentar y tolerantes a la leche caliente, mientras que 41 (29,7%) habían superado la alergia. El nivel medio de expresión de FOXP3 en el grupo CMA fue de 2,07 ± 1,23; inferior al obtenido en el grupo control de 2,98 ± 1,52 (p<0,001). Un valor por debajo de 1,45 indica alergia. El nivel sérico medio de vitamina D en el grupo de estudio (29,67 ± 7,09 ng/ml) fue más bajo que en el grupo control, 33,35 ± 4,13 ng/ml (p<0,001). No se encontraron diferencias significativas en el contenido medio de vitamina C en suero.
Conclusiones: El aumento de la expresión de FOXP3 mRNA puede predecir la adquisición de tolerancia más rápida en los bebés con CMA. Estos niños tienen niveles séricos más bajos de vitamina D que los niños sanos. No se encontró relación entre la historia natural de CMA y la concentración de vitamina C en suero.
Palabras clave: Alergia alimentaria. FOXP3. Tolerancia. Niños. Vitamina D. Vitamina C. Leche de vaca.

Introduction

Cow's milk allergy (CMA) is the most common form of food allergy, with an estimated prevalence in children of between 0.5% and 3% [1-3]. Although recent studies indicate that CMA typically gives way to atopic dermatitis, it is also associated with an elevated risk of developing allergy to other foods, as well as asthma and allergic rhinitis [4]. Most importantly, CMA increases the risk of anaphylaxis, worsens quality of life, and may be fatal. Although most children develop tolerance to cow's milk protein (CMP) before the fourth-fifth year of life, some fail to do so [5,6]. Tolerance to allergens may be acquired naturally or through immunotherapy [7]. A comprehensive understanding of the mechanisms promoting tolerance to CMP may enable the development of new approaches for prevention and treatment [8].

Although the pathogenic mechanism underlying the natural acquisition of tolerance to foods, is not precisely understood, a significant role in this process is played by antigen-specific $CD4^+CD25^+$ Treg lymphocytes [9], which regulate the immune response by inhibiting the activity of T_H1 and T_H2 lymphocytes [10]. The best marker for Treg lymphocytes is the transcription factor forkhead box P3 (FOXP3) [11].

The acquisition of food tolerance is influenced by a number of factors, including genetic predisposition, age, maturity of the intestine and its microbiota, type of feeding, exposure to allergens, maternal and infant diets, and various external factors [12,13]. Children with CMA acquire tolerance to CMP faster and more frequently than children with fish or peanut allergy [14]. Around 75% of children with CMA tolerate processed dairy products, ie, products subjected to heat treatment, but do not tolerate them in their "raw" state [15]. Children who tolerate baked milk tend to display a milder course of food allergy [15]. These findings point to 2 different phenotypes of CMA: heated milk–reactive and heated milk–tolerant. Food allergy is also affected by specific IgE antibodies (sIgE), with high sIgE concentrations in serum being indicative of a longer time to acquisition of tolerance [1,16].

Dietary factors play a significant role in the development of tolerance, either directly via the immune system or indirectly via the microbiota [17]. Although epidemiological data regarding the protective effect of foods against the development of allergic diseases is weak, the consumption of fruit and vegetables—particularly those containing vitamins A, D, and E and the mineral zinc—may have protective effects [18]. Indeed, low consumption of vitamin E and antioxidants by pregnant women can lead to the development of asthma in their children [19]. Vitamin C stimulates prostaglandin and cytokine synthesis and inhibits histamine activity and expression of proinflammatory cytokines (IL-6, TNF-α). It also influences the promotion of T_H1 lymphocytes and inhibits the action of T_H2 lymphocytes. It has been shown that consumption of vitamin C by women during pregnancy reduces the risk of occurrence of wheezing and eczema in their children [20]. The active form of vitamin D3 induces the inherent immune response, stimulates Treg cell development by the action of dendritic cells, and, most importantly, activates tolerogenic dendritic cells [21], which regulate the activity of immunoglobulin-like transcript 3 and 4 inhibitor receptors [22]. The expression of these receptors results in the inhibition of the activation of NF-κB, an enhancer of activated B cells [22]. A number of studies indicate a relationship between vitamin D3 consumption and the development of food allergy [22,23].

Based on these considerations, the aim of the present study was to determine how FOXP3 mRNA expression and serum vitamin C and D concentration affect tolerance in children aged <2 years with CMA.

Materials and Methods

A prospective 2-stage study was performed during the period 2014-2016. It included 536 infants with suspected CMA from the Clinic of Pediatric Allergology, Gastroenterology and Nutrition, Medical University of Lodz, and the Clinic of Pediatrics, Allergology and Gastroenterology, CM Bydgoszcz NCU.

Stage I: The inclusion criteria for the first stage of the study comprised age <7 months and a history of parent-reported adverse reactions to milk. Based on the results of an elimination diet, 304 children qualified for oral food challenge (OFC), which was performed according to current recommendations [24-26]. The final study group comprised 138 infants with confirmed CMA, while the control group included 101 healthy infants from an outpatient clinic without sensitization, allergy symptoms, or a diagnosis of food allergy from a physician. This stage, ie, when the children were qualified for OFC, included the following laboratory tests:

- Determination of sIgE antibodies to cow's milk, hen's egg, soy, wheat, gluten, fish, peanuts, nuts, potato, apple, peach, and carrot using the Polycheck method (BioCheck GmbH); the lower detection limit was <0.35 kU/L.
- Serum 25(OH)D concentration was determined using a 1-step delayed chemiluminescent microparticle immunoassay (ARCHITECT 25-OH Vitamin D 5P02, Abbott Diagnostics). A concentration of ≥30 ng/mL was considered sufficient for the Polish population.
- The blood concentration of vitamin C was evaluated colorimetrically using phosphotungstic acid reagent, according to Kyaw with modifications. The reference range for vitamin C was 6-20 µg/dL.
- Evaluation of FOXP3 mRNA expression was in 3 stages:

Cell isolation: Peripheral blood nucleated cells were isolated from the patient using a Vacutainer vacuum system with the addition of the anticoagulant ethylenediamine tetraacetic acid. Isolation conformed strictly to the study density gradient according to the standard protocol provided with the isolation system. The isolation was carried out using Histopaque 1077 (Sigma-Aldrich) with a density gradient of 1.077. RNA was isolated using the modified Chomczynski method. TRI reagent (Invitrogen) was used for proper isolation, and the process was conducted according to the standard protocol. The concentration of RNA obtained was measured using a supersensitive NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific).

Reverse transcription polymerase chain reaction experiments: The reverse transcription reaction was performed using the High Capacity cDNA Archive Kit (Applied

Biosystems). Complementary DNA was prepared from 1 mg of mRNA, with random hexamer primers, according to the manufacturer's instructions (10 minutes at 25°C, 2 hours at 37°C, and 4°C thereafter) on a polymerase chain reaction gene thermocycler (Applied Biosystems). The resulting cDNA was diluted to a final concentration of 5 ng/mL and used as a matrix for further experiments.

Expression experiments: FOXP3 gene expression was analyzed using a commercially available human assay for Hs01085830_m1 and human ß-actin (Applied Biosystems), which were controlled by an internal reaction while allowing reliable determination of absolute values and the expression of *FOXP3* genes. The analysis was carried out in a genetic

real-time polymerase chain analyzer (7900HT; Applied Biosystems). Comparative analyses of each of these genes in individual patients were performed using specialized computer programs (SDS 2.3 and RQ 2.1; Applied Biosystems). All amplifications were carried out at least twice. The mRNA expression levels of each gene were calculated using the 2-Δ comparative threshold cycle method, as detailed by the manufacturer (Technical Bulletin 2; Applied Biosystems).

Stage II: After a period of 6 consecutive months from stage I, ie, before completion of the second year of life, all children underwent a standardized OFC, first with an extensively heated milk product (baked milk) and then with unheated products. The baked milk challenge was performed with muffins containing 1.3 g of milk protein according to Nowak-Węgrzyn et al [15]. Briefly, the muffin was baked at 180ºC for 30 minutes in an oven and administered in 4 equal portions over a period of 1 to 2 hours in patients older than 12 months, while those aged between 6 and 12 months of age were fed 8 equal portions over a period of 2 hours. OFC with unheated milk was performed as described previously [24-26].

Following challenge with the heated milk, the patients were categorized as heated milk–reactive (ie, no tolerance) or heated milk–tolerant depending on their reaction. Following another challenge with unheated milk, they were also classified as unheated milk–reactive (ie, heated milk–tolerant or partially tolerant) or as allergen-tolerant (ie, outgrown). The procedure is illustrated in a flowchart (Figure 1). The study was approved by the institutional ethics committees (No. 131 RNN/101/14/KE and KB 578/2015), and informed consent was obtained before enrolment.

Statistical Analysis

To identify statistical trends in the data collected, the tests used were the Kruskal-Wallis ANOVA with the post hoc Dunn test, Mann-Whitney test, Spearman rank correlation coefficient, and the χ^2 test of independence with a Yates correction. mRNA expression was examined against milk allergy status using receiver operating characteristic (ROC) curve analyses. Cut-offs were determined for prediction of CMA for *FOXP3* mRNA expression with optimal accuracy. All calculations were performed using STATISTICA v.12 (Statsoft Poland).
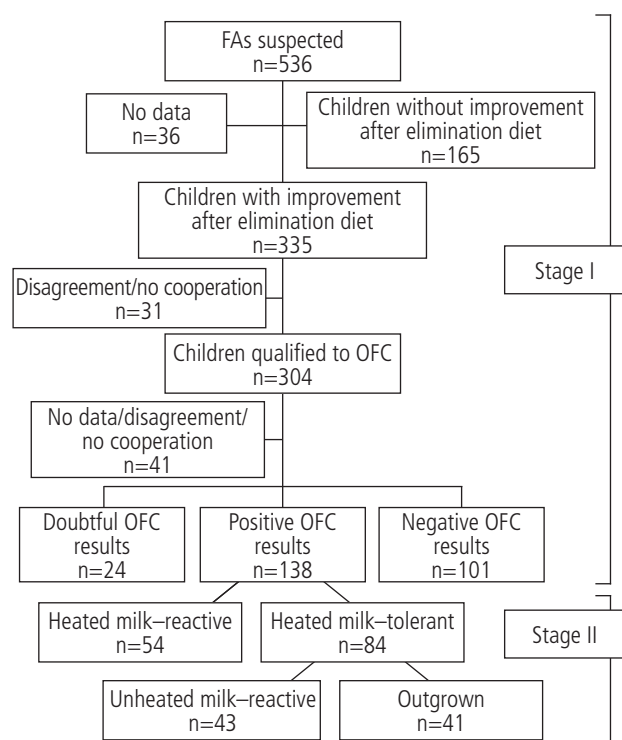
Abbreviations: FA, food allergy; OFC, oral food challenge.
The first stage of the study shows how the children with cow's milk allergy (CMA) were selected from the initial group of children with suspected CMA. The study group was formed from children with positive OFC results (ie, a diagnosis of CMA). The control group included healthy infants without symptoms, sensitization, or a physician's diagnosis of food allergy from an outpatient clinic. The blood was collected for analysis of sIgE, *FOXP3*, and vitamins D and C at the first stage of the study, when the children were qualified for OFC. The blood was frozen and then analyzed in children with confirmed CMA.
The second stage shows the results of OFC with heated (baked) milk, which allowed heated milk–reactive children (no tolerance, ie, persistent CMA) to be distinguished from heated milk–tolerant children. The heated milk–tolerant children were challenged with unheated milk; this enabled unheated milk–reactive children (heated milk–tolerant, ie, partial tolerance) to be distinguished from those who had acquired tolerance to unheated milk (ie, outgrown).

Figure 1. Study design. Flow chart depicting steps involved in patients in this study.

Table 1. Characteristic of the Study and Control Groups at Enrolment in the Study

Parameter	Study Group n=138	Control Group n=101	*P* Value
Sex, No. (%)			
Female	64 (46)	49 (49)	.740
Male	74 (54)	52 (51)	
Age, mo			
Median (IQR)	5 (5)	6 (9.5)	
Q1-Q3	3-8	3.5-13	.052
Mean (SD)	7.4 (7.2)	8.5 (6.2)	
Place of residence, No. (%)			
Nonrural	93 (67)	71 (70)	.630
Rural	45 (33)	30 (30)	

Results

Table 1 presents the characteristics of the study and control groups.

Among children aged 2 years, OFC with heated and unheated milk products showed 54 children (39.1%) to be heated milk–reactive (ie, with persistent CMA) and 84 (60.9%) to be heated milk–tolerant. Following the challenge with unheated milk, 43 children (31.2%) were found to be unheated milk–reactive and heated milk–tolerant (ie, with partial tolerance), while 41 (29.7%) were classified as allergen-tolerant (ie, they had outgrown their allergy) (Figure 1).

A relationship was found between acquisition of tolerance and sex. Girls outgrew CMA more quickly than boys: while 25 girls (40%) with CMA developed complete tolerance, this was the case for only 16 boys (21.6%) (P=.025). No such relationship was found for the heated milk–reactive children (girls n=20 [31.3%] vs boys n=34 [45.9%]) or the unheated milk–reactive children (girls n=19 [29.7%] vs boys n=24 [32.4%]; P>.05).

We analyzed acquisition of tolerance with respect to the time of onset of CMA. It was found that 54 (39.1%) children continued to demonstrate symptoms in their second year of life, with the mean onset of the disease occurring at 4 (1.9) months (median, 5; range, 1-7 months). Children who outgrew their allergy (n=41) or developed tolerance to baked milk (n=43) had symptoms at 2.3 (1.5) months of life (median, 2; range, 1-6 months) and 2.4 (1.6) months of life (median, 2; range, 1-6 months), respectively; this was significantly earlier than the children developing persistent CMA (n=54) symptoms at 4.1 (1.9) months of life (median, 5; range, 1-7 months; P=.001). Children who outgrew their allergy reported significantly more frequent symptoms between the first and third months of life than those in whom symptoms appeared later in life (P=.012). CMA was more likely to persist to the second year of life in children in whom the onset was at the age of 6-7 months than in those with an earlier onset (P=.001). No significant relationship was found between the acquisition of tolerance to baked milk and age of onset of CMA symptoms (P>.05).

Clinical symptoms solely associated with the skin (rash, atopic dermatitis, urticaria, itching, angioedema) were recorded in 32 children (23.19%). Forty-three children (31.16%) presented symptoms associated with the digestive tract (diarrhea, constipation, colic, flatulence, regurgitation, vomiting, blood/mucus in stools, or poor weight gain). Multiple organ symptoms were observed in 63 children (45.65%). No differences were found between the development of tolerance to CMP and the occurrence of symptoms associated with the skin or digestive tract (P>.05).

At 2 years of age, 11 children (26.83%) had outgrown CMA, which had manifested as skin symptoms, and 13 (30.23%) tolerated baked milk; however, 8 children (14.8%) continued to present symptoms. Of those aged 2 years with symptoms primarily associated with the digestive tract, 13 (24.07%) continued to have allergy, while 16 (39%) had acquired full tolerance. No relationship was found between the presence of symptoms associated with the digestive tract and the development of tolerance (P>.05). Interestingly, a significantly greater percentage of children who outgrew

Table 2. Specific IgE Concentration According to Type of Acquisition of Tolerance in Children With CMA

	Mean (SD)	Median Range	P Value
Heated milk–reactive (persistent CMA= no tolerance) n=37 (71.2%)	6.5 (4.9)	5.3 0.61-19.2	.75
Unheated milk–reactive (partial tolerance) n=9 (17.3%)	2.3 (1.6)	2.4 0.56-5.2	.01[a]
Outgrown (full tolerance) n=6 (11.5%)	6.1 (2.3)	6.5 3.3-9.6	.04[b]

Abbreviation: CMA, cow's milk allergy.
[a]No tolerance vs partial tolerance.
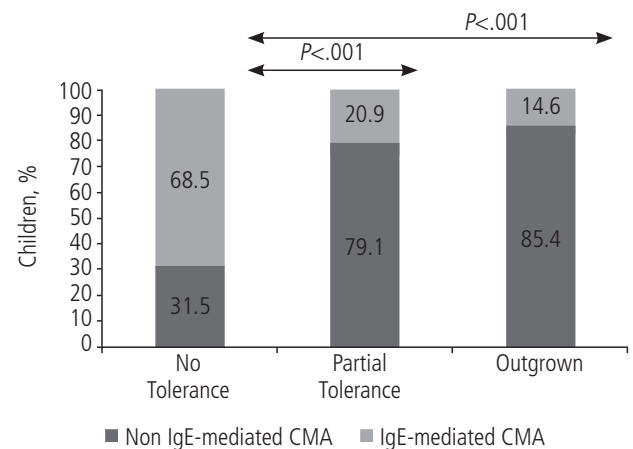[b]Partial tolerance vs outgrown.

Figure 2. Percentage of children with IgE-mediated and non–IgE-mediated CMA regardless of type of acquisition of tolerance (ie, no tolerance, partial tolerance, heated milk–tolerant, unheated milk–reactive, outgrown).

CMA presented single-organ symptoms (n=27; 65.85%) compared with those who were reactive to heated milk (n=21; 38.89%) (P=.009). Similarly, children with persistent CMA were significantly more likely to develop partial tolerance to CMP if they presented single-organ symptoms (27; 62.79%) than if they presented multiple-organ symptoms (16; 37.21%) (P=.019).

sIgE for cow's milk was detected in 52 infants with CMA (37.7%). Only 6 (11.5%) developed tolerance to raw milk, ie, they outgrew CMA, by the second year of life. Most of the infants with IgE-mediated CMA (n=37; 71.2%) did not acquire tolerance, ie, they even reacted to baked milk. Significantly more nonatopic children than atopic children developed tolerance to raw milk or baked milk (P=.001; P=.001) (Figure 2). The mean concentration of milk IgE in the heated milk–reactive children, as well as in those who outgrew CMA, was significantly higher than in children with partial tolerance (P=.01; P=.04 respectively) (Table 2).

Table 3. *FOXP3* Gene Expression in the Groups Studied According to Sex[a]

Groups Studied by Sex	*FOXP3* Gene Expression			*P* Value
	Mean (SD)	Median	Range	
Study group				
Girls, n=64 (46%)	2.25 (1.21)	2.18	0.06-4.83	.08
Boys, n=74 (54%)	1.92 (1.24)	1.74	0.01-5.59	
Heated milk–reactive (persistent CMA=no tolerance)				
Girls, n=20	1.78 (1.31)	1.39	0.06-4.06	.29
Boys, n=34	1.33 (1.11)	1.13	0.01-4.23	
Unheated milk–reactive (partial tolerance)				
Girls, n=19	2.2 (0.87)	2.15	0.06-3.89	.92
Boys, n=24	2.2 (1.13)	2.24	0.51-5.59	
Outgrown (full tolerance)				
Girls, n=25	2.68 (1.23)	2.82	0.13-4.83	.95
Boys, n=16	2.73 (1.05)	2.78	0.98-4.25	
Control group				
Girls, n=49 (49%)	2.85 (1.58)	2.47	1.03-6.86	.21
Boys, n=52 (51%)	3.09 (1.46)	2.75	0.56-6.49	

[a]Outgrown girls vs girls with persistent CMA, *P*=.03; outgrown boys vs boys with persistent CMA, *P*=.001; boys with persistent CMA vs boys with partial tolerance, *P*=.013; girls in the study group vs girls in the control group, *P*=.15; boys in the study group vs boys in the control group, *P*=.0001.
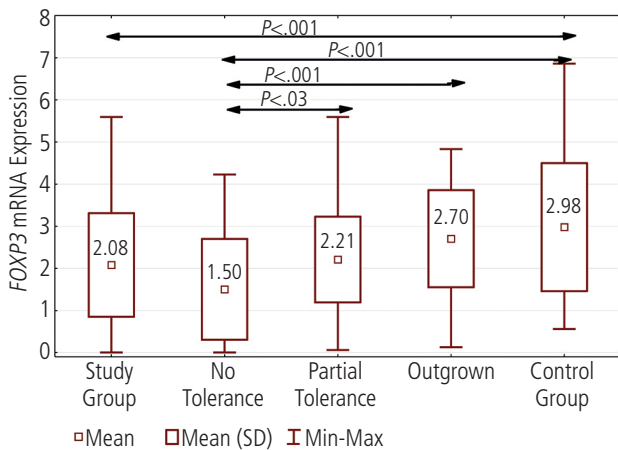

Figure 3. Acquisition of tolerance according to the level of *FOXP3* mRNA expression (mean, mean [SD], min-max) in the study and control groups. The study group comprised children with CMA, who were observed for 6 months before performance of the OFCs. The OFC results enabled children with persistent CMA (ie, not tolerant) to be distinguished from those with partial tolerance (ie, heated milk–tolerant and unheated milk–reactive) and those who had outgrown CMA (ie, full tolerance).

The mean level of *FOXP3* expression in the study group was 2.07 (1.23); this was significantly lower than in the control group (2.98 [1.52]; *P*<.001). The heated milk–reactive children also displayed significant differences in mean *FOXP3* expression compared with children who outgrew CMA, those who developed partial tolerance, and the control group (Figure 3). Significantly greater *FOXP3* expression was detected in children with non–IgE-mediated CMA than in those with IgE-mediated CMA (2.25 [1.16] vs 1.79 [1.29]; *P*=.03). Lower sIgE levels were associated with higher *FOXP3* expression in the study group (ρ=−0.344; *P*=.01); however, no significant correlation was found between different types
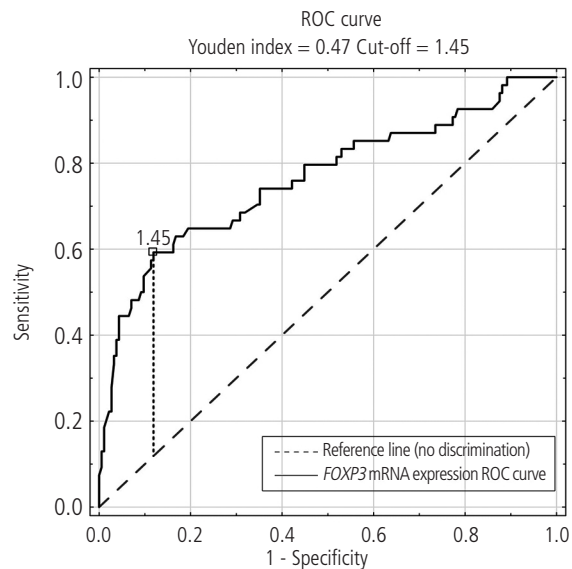

Figure 4. Receiver operating-characteristic (ROC) curve and area under the curve (AUC) of the level of *FOXP3* mRNA expression for diagnosing persistent CMA in the second year of life.
The cut-off value for the level of *FOXP3* mRNA expression distinguishing between children with persistent CMA and tolerant children (ie, partial and full tolerance) in the second year of life was 1.45 (Youden index, 0.474). The AUC of the ROC curve of the level of *FOXP3* mRNA expression for diagnosing persistent CMA in the second year of life was 0.764 (Youden index, 0.041; *P*<.0001).

of acquisition of tolerance. The relationship between *FOXP3* gene expression and sex is presented in Table 3.

After distinguishing between children with persistent CMA and heated milk–tolerant children (ie, those with partial and full tolerance) in the second year of life, the cut-off value for the level of *FOXP3* mRNA expression was identified as 1.45. This indicates that children with a level of *FOXP3* mRNA expression <1.45 are more likely to have persistent CMA in the second year of life.

ROC analysis found that the level of *FOXP3* mRNA expression displayed good specificity (88%) but weak sensitivity (59%) for identifying patients with persistent CMA.
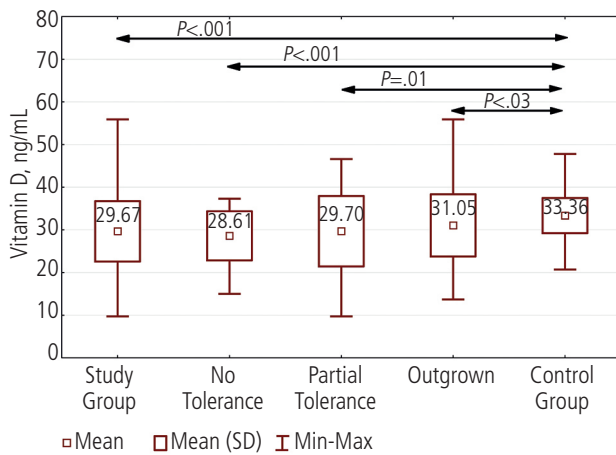

Figure 5. Acquisition of tolerance according to the serum level of vitamin D (mean, mean [SD], min-max) in the study and control groups. The study group comprised children with CMA, who were observed for 6 months before performance of the OFCs. The results enabled children with persistent CMA (ie, no tolerance) to be distinguished from those with partial tolerance (ie, heated milk–tolerant and unheated milk–reactive) and those who had outgrown CMA (ie, full tolerance).
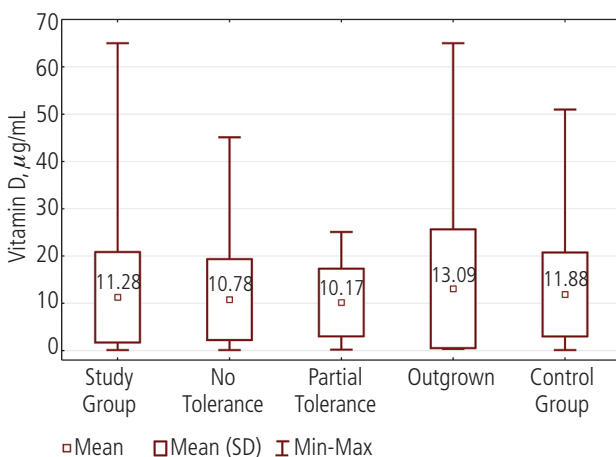

Figure 6. Acquisition of tolerance according to serum level of vitamin C (mean, mean [SD], min-max) in the study and control groups. The study group comprised children with CMA who were observed for 6 months before performance of OFCs. The results enabled children with persistent CMA (ie, no tolerance) to be distinguished from those with partial tolerance (ie, heated milk–tolerant and unheated milk–reactive) and those who had outgrown CMA (ie, full tolerance)..

Therefore, although the cut-off value identified seems to be a good marker when screening for children with persistent CMA, it may incorrectly classify children without CMA (Figure 4).

The mean serum level of vitamin D in the study group (29.67 [7.09] ng/mL), including children with persistent CMA, those with partial tolerance, and those who outgrew CMA by the second year of life, was significantly lower than in controls (33.35 [4.13] ng/mL) (*P*<.001). However, no significant differences were observed within the study group itself (Figure 5).

No significant differences in mean serum vitamin C content were found between children from the study group (11.28 [9.38] µg/dL), including those with no tolerance, those with partial or full tolerance, and children from the control group (11.89 [8.87] µg/dL) (*P*>.05) (Figure 6).

In the study and control groups, no significant relationship was found between *FOXP3* expression and vitamin C or D level or with the way tolerance was acquired. A significant relationship was found between the concentrations of *FOXP3* and vitamin C, but only in children with partial tolerance ($\rho=-0.343$; *P*=.02).

Discussion

Most of the children with CMA in the study group (60%) acquired tolerance to heated milk by the second year of life: one third of the study group tolerated heated milk, but not fresh milk, while another third outgrew their allergy. Our findings suggest that evaluating the expression of *FOXP3* mRNA may assist in estimating the probability of acquiring tolerance to CMA in infants.

Data regarding the time to acquisition of tolerance to milk is varied. Most studies indicate that children with CMA typically acquire tolerance by around the age of 3 to 7 years; however, CMA may persist even to adulthood [2,5,6]. Consistent with our findings, the results of the EuroPrevall cohort study indicate that it is possible to acquire tolerance by the age of 2 years [3]. Until recently, the only method of managing CMA was strict elimination of CMP. Observational studies indicate, however, that most children tolerate CMP in modified forms, such as after heat treatment [15], and children with CMA outgrew their allergy more quickly when exposed to heated allergens in food [15,27]. These findings were true both for children with IgE-mediated CMA and for those with non-IgE-mediated CMA [28].

The development of tolerance is also dependent on the pathogenic mechanism underlying the allergy. Consistent with previous studies, our findings indicate that children with non–IgE-mediated CMA develop tolerance more quickly than those with IgE-mediated CMA. Vanto et al [16] report that 63% of children with IgE-mediated CMA and 96% with non–IgE-mediated CMA were able to tolerate CMP by the fourth year of life. However, findings regarding the importance of the sIgE concentration in CMA are varied: while sIgE concentration does not determine the severity of the reaction, the presence of high levels of sIgE in the blood is associated with a longer time to acquisition of tolerance [5,12,29]. Our findings indicate a lower concentration of sIgE in heated milk–tolerant children than in heated milk–reactive children, although the children

who outgrew CMA had a higher concentration of sIgE than those who were unheated milk–reactive.

Recent studies emphasized the importance of even low levels of sIgE. The presence of sIgE against milk or eggs, even at levels <0.35 kU/L in children, has been associated with an increased risk of persistence of sensitivity to food allergens, as well as the development of sensitivity to inhaled allergens and atopic dermatitis at the age of 5 years [30]. A higher level of sIgE increases the likelihood of allergic reactions to food, as well as the strength of the clinical symptoms, in terms of both their type and frequency of occurrence [31].

Food allergy typically manifests as symptoms affecting multiple organs. Our findings indicate that significantly fewer children with symptoms affecting multiple organs developed tolerance to raw milk and baked milk than those with symptoms affecting a single organ, suggesting that symptoms affecting multiple organs may be more closely associated with a food allergy phenotype.

We also found that the children who outgrew CMA manifested allergic symptoms significantly earlier—often during the first months of life—than those who developed them later in life. In heated milk–reactive children, onset of symptoms was typically after 6 months of life. This may be associated with the natural development of sIgE. In the first months of life, non–IgE-mediated CMA is more predominant, and prognosis is better.

Although gender has been found to be associated with the clinical course of asthma, its influence on food allergy and the acquisition of tolerance remain unknown. The present findings indicate that more girls than boys were affected by allergies; hormones may play a role in allergy development [32].

Consistent with findings from previous studies [33-35], we found that the lowest level of *FOXP3* mRNA expression was observed in children with persistent CMA during the second year of life; higher levels were observed in heated milk–tolerant children, and the highest levels in children without allergy. These observations may be associated with Treg being exhausted by exposure to the allergen. In addition, the loss of the ability to regenerate Treg pools caused by such contact may play a key role in determining the development of allergy or sensitivity [36]. Our findings indicate that determination of *FOXP3* mRNA expression could prove useful for identifying subjects at increased risk of persistent CMA: a *FOXP3* mRNA expression value <1.45 suggests that these children are more likely to have persistent CMA. Bullens et al [37] report that a cord blood *FOXP3*/CD3γ mRNA ratio below 0.32 predicted an allergic outcome.

FOXP3 level is also related to total IgE level [34]. Our results confirmed an inverse correlation between sIgE level and *FOXP3* expression, which is in line with data reported by Matsumoto et al [38].

Perezabad et al [39] and Chambers et al [40] indicated that the levels of *FOXP3*, Treg lymphocytes, and vitamin D were significantly lower among children with food allergy than in healthy controls. Most importantly, it was found that the level of vitamin D3 in serum correlated with the numbers of Foxp3+ Treg cells in the peripheral blood of children with asthma [40]. While we found no association between *FOXP3* mRNA expression and vitamin D concentration, a significantly

lower level of vitamin D was found in children with CMA than in controls; however, as noted by Molloy et al [41], no relationship was found with the acquisition of tolerance.

Antioxidant intake may reduce the risk of allergic disease by protecting against oxidative tissue damage. Major sources of antioxidants in the Western diet are fruits, vegetables, and vitamin C [42]. However, we found no significant correlation between vitamin C level and acquisition of tolerance. Similarly, no consistent evidence has previously been found for an association between the occurrence of asthma and fruit and vegetable intake among asthma patients [43].

Our study is limited by the fact that we analyzed *FOXP3* mRNA, vitamin D, vitamin C, and sIgE at a single time point. Given the possible variation in the concentration of the parameters tested over time, it would be interesting to follow the level at various time points in future studies.

Becoming better acquainted with the mechanisms leading to the acquisition of natural tolerance to CMP offers hope for the possibility of designing new models for the prevention and treatment of food allergy; however, further studies are required. Dawicki et al [44] suggested that induction of *FOXP3* regulatory T cells might be a useful strategy for induction of tolerance in food-allergic patients.

In conclusion, the results of this study suggest that increased *FOXP3* mRNA expression can predict faster acquisition of tolerance in infants with CMA. Regardless of whether they acquire tolerance, children with CMA have lower serum vitamin D levels than healthy children. No relationship was found between the natural history of CMA in the tested 2-year-olds and serum vitamin C concentration.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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